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Stability of polyphenols in chokeberry (*Aronia melanocarpa*) subjected to *in vitro* gastric and pancreatic digestion

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Abstract

Berries and red fruits are dietary sources of polyphenols with reported health benefits. As part of the diet, polyphenols are ingested as complex mixtures immersed in a food matrix which is digested in the gut. Epithelial cells lining the gut are regularly exposed to these digested mixtures. To understand the effects of dietary polyphenols on human gut health it is essential to determine their stability and fate in the lumen. In this work, we investigated the effects of an *in vitro* gastric and pancreatic digestion on the stability and composition of the major polyphenols in chokeberry juice. Digestion was carried out with a mixture of pepsin-HCl for 2 h, followed by a 2 h incubation with pancreatin and bile salts at 37 °C with shaking, in the absence of light and under N_2. After digestion, the chokeberry samples were acidified, filtered and HPLC-DAD/HPLC–MS–MS analysed to determine the content of total soluble recovered phenolics. Gastric digestion had no substantial effect on any of the major phenolic compounds in chokeberry, namely anthocyanins, flavan-3-ols, flavonols and caffeic acid derivatives. However, these compounds were significantly altered during the pancreatic digestion and this effect was more marked for anthocyanins (approximately 43% was lost during the 2 h treatment with pancreatin). Flavonols and flavan-3-ols decreased by 26% and 19%, respectively. Neochlorogenic acid decreased by 28% whereas chlorogenic acid was increased by 24%. *In vitro* digestion of standard phenolic compounds, representing each of the groups of phenolics in chokeberry, confirmed some of the observed changes. Interactions with the digestive enzymes were not responsible for the observed losses which were mostly due to the chemical conditions during pancreatic digestion. Our results, in accordance with previously published results, show that dietary polyphenols are highly sensitive to the mild alkaline conditions in the small intestine and that a good proportion of these compounds can be transformed into other unknown and/or undetected structural forms with different chemical properties and, consequently, different bioaccessibility, bioavailability and biological activity.

Keywords: *In vitro* digestion; Chokeberry; *Aronia melanocarpa*; Polyphenols; Anthocyanins; Flavonols; Flavan-3-ols; Chlorogenic acid

1. Introduction

Among other fruits, berries and red fruits are important dietary sources of polyphenols, such as anthocyanins, flavan-3-ols, procyanidins, flavonols and cinnamic acid derivatives (Bérmudez-Soto & Tomás-Barberán, 2004; Wu, Gu, Prior, & McKay, 2004). For the past several years, bioavailability studies in humans have given evidence of the absorption of many of these dietary polyphenols and have shown that uptake varies widely, depending on the type of compound and/or food matrix (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). Although for some compounds, such as anthocyanins, concentration values in plasma or urine may have been underestimated due to instability of the compounds and sample processing (Felgines et al., 2003), absorption of dietary polyphenols is, in general, very low (plasma concentrations of total metabolites are in the nM to low μM range) and the time to reach maximum concentration ranges between 30 min and several hours, depending on the site of absorption (Manach

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et al., 2005). Also, the bioavailability of polyphenols to peripheral tissues can be diminished by high intestinal and biliary secretion of their conjugates (Silberberg et al., 2006). Therefore, the bulk of dietary polyphenols and their conjugates must remain in the gut lumen and it is the gastrointestinal tract where these compounds may exert important beneficial effects, such as inhibition of abnormal cell proliferation and protection against cancer development. A chemopreventive role for berries has been shown in the colon and esophagus of animals (Kang, Seeram, Nair, & Bourquin, 2003; Kresty et al., 2001) and a number of studies have reported antiproliferative effects on human colon cancer cells exerted by berry extracts, berry fractions or by specific berry phenolics, such as anthocyanins (Olsson, Gustavsson, Andersson, Nilsson, & Duan, 2004; Malik et al., 2003; Bagchi, Sen, Bagchi, & Atalay, 2004; Seeram, Adams, Hardy, & Heber, 2004; Zhao, Giusti, Malik, Moyer, & Magnuson, 2004; Katsube, Iwashita, Tsushida, Yamaki, & Kobori, 2003).

To further understand the possible beneficial effects of fruit polyphenols on human health and in particular, on gut health, it is essential to determine their metabolic fate. As part of the diet, polyphenols are ingested as complex mixtures immersed in a food matrix, e.g. fruit or fruit juice, which undergo a digestion process in the gut. It is important to determine how this digestion process affects polyphenols and their stability as this, in turn, will affect their bioaccessibility for uptake as well as their possible beneficial effects on the cells lining the gut. At this time, there is little information describing the *in vivo* effects of the digestion process on dietary polyphenols. The stability and metabolism of some dietary procyanidins in human gastric contents (Rios et al., 2002), of quercetin in human jejunum (Petri et al., 2003) and of anthocyanins in rat intestine (He, Magnuson, & Giusti, 2005) and in pig gastrointestinal tract (Wu, Pittman, & Prior, 2006) have recently been reported. One more common and simple approach is the application to foods or food components of *in vitro* methods that simulate the digestion conditions. There are various reports on the effects of *in vitro* digestion on dietary polyphenols, e.g. flavonoids and phenolic compounds in orange juice (Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán, 2001), pomegranate juice (Perez-Vicente, Gil-Izquierdo, & Garcia-Viguera, 2002) and broccoli (Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera, 2004), flavan-3-ols in cocoa (Zhu et al., 2002), anthocyanins in raspberry (McDougall, Dobson, Smith, Blake, & Stewart, 2005), quercetin in onions and apples (Boyer, Brown, & Liu, 2005), isoflavones in soy bread (Walsh, Zhang, Vodovotz, Scwartz, & Failla, 2003) or myricitrin (Yokomizo & Moriwaki, 2005). Recently, the stability and biotransformations of anthocyanins under *in vitro* incubation with human fecal suspension has also been reported (Aura et al., 2005; Fleschhut, Kratzer, & Rechkemmer, 2005).The results obtained with these studies have shown that *in vitro* methods are very useful for evaluating the effects of factors such as food matrix, digestion conditions or interaction with other compounds, on the stability and properties of polyphenols which may affect their bioaccessibility and final metabolic fate.

In the present study we investigated the digestive stability of the major polyphenols in a commercial berry juice using an *in vitro* model that simulates some of the conditions (pH, temperature and enzymes) in the stomach and in the duodenum. Chokeberry (*Aronia melanocarpa*) originates from North America and has been extensively cultivated in Denmark, eastern Europe and Russia. It is widely used in the food industry, either on its own, or blended with other fruits (e.g. in juice and soft drink-making, wine production, food colouring, natural health products) and was selected for this particular study because it is very rich in polyphenols, mostly anthocyanins and procyanidins (Bérmudez-Soto & Tomás-Barberán, 2004; Wu et al., 2004), and has demonstrated antiproliferative and anticarcinogenic effects on human colon cancer cells (Olsson et al., 2004; Malik et al., 2003; Bagchi et al., 2004; Zhao et al., 2004).

2. Materials and methods

2.1. Materials

Chokeberry (*Aronia melanocarpa*) commercial juice concentrate (1.3 g ml^{-1}) was kindly provided by the juice manufacturing company Juver Alimentación S.A. (Murcia, Spain), divided into aliquots and stored at −20 °C until used. Chlorogenic acid and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercetin-3-rutinoside and cyanidin-3-rutinoside were supplied by Merck (Darmstadt, Germany) and by Polyphenol (Sandnes, Norway), respectively. Ultrapure Millipore water was used for all solutions. Solutions of cyanidin-3-rutinoside (50 mg/l), (+)-catechin (300 mg/l) and chlorogenic acid (100 mg/l) were prepared in water. Quercetin-3-rutinoside was dissolved in 200 μl of DMSO and diluted in water (200 mg/l).

2.2. In vitro digestion

For each *in vitro* incubation, an aliquot of frozen chokeberry concentrate was thawed and diluted (1:5, chokeberry: water) to approximately 15 ° Brix (20 °C) and a final pH of 3.4. This diluted chokeberry juice was subjected to successive *in vitro* gastric and pancreatic digestion, following a previously published method (Gil-Izquierdo et al., 2001) with some modifications. For the gastric digestion, samples of the chokeberry juice were adjusted to pH 2.0 with concentrated HCl and the mixture was incubated with 15750 IU of pepsin (from porcine stomach mucosa, pepsin A, EC 3.4.23.1, Sigma, Steinheim, Germany) in a shaking bath for 2 h (final incubation volume 50 ml). At the end of the gastric digestion, samples were taken for analysis of the phenolic compounds. A 20 ml sample of the pepsin digest was then neutralized with $NaHCO_3$ before addition

of 5 ml of pancreatin (from porcine pancreas, 4 g/l, 1.6×10^{-3} US Pharmacopeia (USP) specifications, Sigma)–bile extract mixture (25 g/l, containing a mixture of sodium cholate and sodium deoxycholate, Sigma) and further incubation of the mixture (pH 7.5) for an additional 2.0 h. In the published protocol (Gil-Izquierdo et al., 2001), segments of a cellulose dialysis tubing were used during the incubation with pancreatin. We determined the effect of using this dialysis membrane on the recovery of soluble phenolics. HPLC analyses showed that the levels of soluble recovered flavonols, caffeic acid derivatives and flavan-3-ols, in chokeberry juice digested without using a dialysis membrane, were significantly higher ($p < 0.01$) than those measured after digestion with the published method. We also examined the effects of light and reduced levels of O_2 on the recovered soluble phenolics and found that anthocyanins recovery was enhanced by 50% when incubations were done in the dark and after flushing with N_2. Consequently, and for all subsequent experiments, we performed the *in vitro* digestions without using the dialysis membrane and both gastric and pancreatic incubations were done in a conical flask at 37 °C with shaking, in the absence of light. To eliminate some of the dissolved O_2, incubation mixtures were flushed with N_2 for 10 min after addition of the enzymes and kept closed. Controls without added enzymes were run in parallel to differentiate the effects due to the presence of enzymes from those that may be caused by the chemical environment in the assays.

After the pancreatic digestion, and to ensure inactivation of enzymes and stability of the phenolic compounds, aliquots of the digested samples were rapidly acidified to pH 2.0 prior to analysis of the phenolic compounds. A total of $n = 6$ *in vitro* incubations with chokeberry juice were carried out and values are presented as means ± SD. Where indicated, mean values were compared using the student-*t* test and were considered significant for $p < 0.01$. Selected commercial standard compounds: cyanidin-3-rutinoside, quercetin-3-rutinoside, (+)-catechin and chlorogenic acid, were subjected to the same *in vitro* digestion. A starting standard solution of 50 ml (at the concentration stated for each compound in the Materials section) was subjected to the gastric digestion. A sub-sample (20 ml) of the gastric digested solution was then further digested with pancreatin. Totally, $n = 3$ *in vitro* incubations were performed and values are presented as means ± SD.

2.3. Analysis of phenolic compounds

Samples taken after gastric and pancreatic digestion were filtered (0.45 μm) and analyzed by HPLC-diode array and HPLC-mass spectrometry (MS)–(MS) to determine the composition and recovery of soluble phenolics (Bérmudez-Soto & Tomás-Barberán, 2004). Phenolic compounds were identified by their UV spectra, by their molecular weight and molecular fragments and, wherever possible, by chromatographic comparisons with authentic synthetic standards. Chromatograms were recorded at 520 nm (anthocyanins), 360 nm (flavonols), 320 nm (hydroxycinnamic acid derivatives) and 280 nm (flavan-3-ols) and quantification was done using cyanidin-3-rutinoside, quercetin-3-rutinoside, chlorogenic acid and (+)-catechin, respectively. Flavan-3-ols were quantified as the sum of a group of small peaks with the same spectral properties of catechin and masses corresponding to monomers, dimers, trimers and tetramers of catechin. The repeatability of the quantitative analysis was ±4% (Bérmudez-Soto & Tomás-Barberán, 2004). We observed that, in the samples taken after pancreatic digestion, a precipitate was formed upon acidification. It is known that certain proteins can bind and precipitate polyphenols (Charlton et al., 2002). We checked for possible losses of phenolics due to co-precipitation by comparing levels of soluble recovered phenolic compounds in acidified *vs.* non-acidified samples. No differences were detected between the two types of samples (data not shown), indicating that there were no detectable losses of these compounds due to precipitation.

3. Results

3.1. In vitro gastric digestion

Individual results for each of the phenolic compounds investigated and percentage of changes during the *in vitro* digestion of chokeberry juice are presented in Table 1. Gastric-simulated digestion of chokeberry juice with pepsin–HCl (pH 2.0) for 2 h did not have a substantial effect on any of the major phenolic compounds in chokeberry juice. The levels of soluble recovered flavonols and caffeic acid derivatives, neochlorogenic acid (3-caffeoylquinic acid) and chlorogenic acid (5-caffeoylquinic acid), were not altered during the stomach treatment. Flavan-3-ols were quite stable, as only a small decrease of ~15% (not significant) in the total levels of these compounds was observed. We also detected a slight increase (~7%; $p < 0.01$) in the quantities of total anthocyanins after the gastric incubation. The results obtained for digested chokeberry juice were further confirmed with pure commercial compounds. Cyanidin-3-rutinoside, quercetin-3-rutinoside, (+)-catechin and chlorogenic acid, used for the quantification of each of the major phenolic groups in chokeberry juice, were subjected to the same *in vitro* digestion process. Results are shown in Table 2. After 2 h of digestion with pepsin under acidic conditions, the flavonol quercetin-3-rutinoside and the caffeic acid derivative chlorogenic acid were fully recovered, whereas pure (+)-catechin decreased slightly (~3%, $p < 0.01$). A small increase in cyanidin-3-rutinoside (approximately by 6%, not significant) was also observed after gastric incubation of this pure compound.

3.2. In vitro pancreatic digestion

Following the stomach incubation, the chokeberry juice was then subjected to a 2 h digestion under conditions

Table 1
Mass spectrometry analysis and individual quantities (total mg per incubation mix) of each of the main soluble phenolic compounds determined in the original chokeberry juice and recovered after gastric and pancreatic *in vitro* digestion

(MS)[a]		(MS/MS)[a]		Compounds	Original	Gastric digestion	Pancreatic digestion	% Loss
m/z^-	m/z^+	m/z^-	m/z^+					
				Anthocyanins				
–	449	–	287	Cyanidin 3-galactoside	18.1 ± 0.30	18.7 ± 0.09	11.0 ± 0.69	39.2
–	419	–	287	Cyanidin 3-glucoside	2.08 ± 0.08	2.19 ± 0.03	1.18 ± 0.11	43.3
–	419	–	287	Cyanidin 3-arabinoside	12.0 ± 0.37	12.5 ± 0.01	6.61 ± 0.49	44.9
–	449	–	287	Cyanidin 3-xyloside	1.45 ± 0.05	1.51 ± 0.05	0.71 ± 0.07	50.8
–	–	–	287	Cyanidin	0.36 ± 0.14	1.62 ± 0.13	n.d.	–
					34.0 ± 0.88	36.5 ± 0.17*	19.5 ± 1.35	42.6
				Flavonols				
721	–	301, 462, 594	–	Quercetin hex-pent	1.12 ± 0.10	1.07 ± 0.04	0.80 ± 0.02	28.6
–	465	–	303	Quercetin 3-galactoside	1.35 ± 0.06	1.32 ± 0.03	1.14 ± 0.03	15.5
–	487	–	325[b]	Quercetin 3-glucoside	1.86 ± 0.45	1.99 ± 0.28	1.51 ± 0.07	19.1
–	633	–	325[b]	Quercetin 3-rutinoside	4.44 ± 0.26	4.18 ± 0.23	3.13 ± 0.14	29.6
–	303	–	–	Quercetin	0.18 ± 0.15	0.19 ± 0.16	n.d.	–
					8.95 ± 0.49	8.75 ± 0.33	6.58 ± 0.24	26.5
				Caffeic acid derivatives				
–	377	–	215[b]	Neochlorogenic acid	15.9 ± 0.65	15.3 ± 0.22	11.4 ± 0.80	28.0
–	377	–	215[b]	Chlorogenic aid	14.8 ± 0.17	14.3 ± 0.18	18.3 ± 0.20	23.9[c]
					30.6 ± 0.51	29.6 ± 0.17	29.7 ± 0.79	2.9
289, 577, 865, 1153				*Flavan-3-ols*	35.5 ± 1.87	30.2 ± 1.57	28.7 ± 2.05	19.3

Values are shown as means ±SD ($n = 6$); n.d.: not detected.
Percentage of total loss at the end of the digestion process.
[a] As determined by Bérmudez-Soto and Tomás-Barberán, 2004.
[b] Na^+ adducts.
[c] % of increase.
* Significant differences ($p < 0.01$) in comparison to the original content.

Table 2
Individual quantities (total mg per incubation mix) of each of the commercial phenolic standard compounds in the initial solution and after gastric and pancreatic *in vitro* digestion

Compound	Original	Gastric digestion	Pancreatic digestion	% Loss
Cyanidin 3-rutinoside	2.64 ± 0.10	2.8 ± 0.01	2.40 ± 0.02*	9.1
Quercetin-3-rutinoside	8.03 ± 0.18	8.04 ± 0.11	7.87 ± 0.05*	3.1
(+)-Catechin	16.0 ± 0.01	15.5 ± 0.03*	6.72 ± 0.10	58.0
Chlorogenic acid	4.50 ± 0.01	4.69 ± 0.007	4.27 ± 0.02*	5.1

Values are shown as means ± SD ($n = 3$).
Percentage of total loss at the end of the digestion process.
* Significant differences ($p < 0.01$) in comparison to the original content.

mimicking those of the duodenum. Results are also presented in Table 1. Overall, the most affected compounds were the anthocyanins as about 43% of these compounds were lost during treatment with pancreatin. Total flavonols and flavan-3-ols in chokeberry juice were decreased by approximately 26% and 19%, respectively, during pancreatic digestion. Under our experimental conditions, the levels of the two main caffeic acid derivatives in chokeberry juice, neochlorogenic acid and chlorogenic acid, were also altered: neochlorogenic acid decreased by 28% whereas chlorogenic acid was increased by approximately 24% (Fig. 1, 1–3; peak A, neochlorogenic acid; peak B, chlorogenic acid). In a similar fashion, the tested commercial standard compounds were less stable under the pancreatic digestion conditions than in the gastric simulation (Table 2). Pancreatic digestion of pure cyanidin-3-rutinoside led to a 9% decrease of this anthocyanin ($p < 0.01$) whereas 58% of the pure (+)-catechin was lost after incubation with pancreatin. The pure quercetin-3-rutinoside was, however, very stable under the pancreatic conditions (only 3 % losses, $p < 0.01$). Commercial chlorogenic acid was quite stable during incubation with the pancreatin-bile salts and the levels of this compound were reduced by only approximately 5% ($p < 0.01$). During the pancreatic incubation of pure chlorogenic acid, a second peak was also formed. The spectral properties and retention time of this new peak matched those of the isomer, neochlorogenic acid (Fig. 1, 3–6; peak A, neochlorogenic acid; peak B, chlorogenic acid). We monitored the decrease in the levels of chlorogenic acid and the formation of neochlorogenic acid during the pancreatic incubation period (Fig. 2) which occurred with a concurrent increase of the final pH of the incubation mixture (from 7.5 to 8.5). No caffeic acid was detected during the *in vitro* pancreatic digestion of chloro-

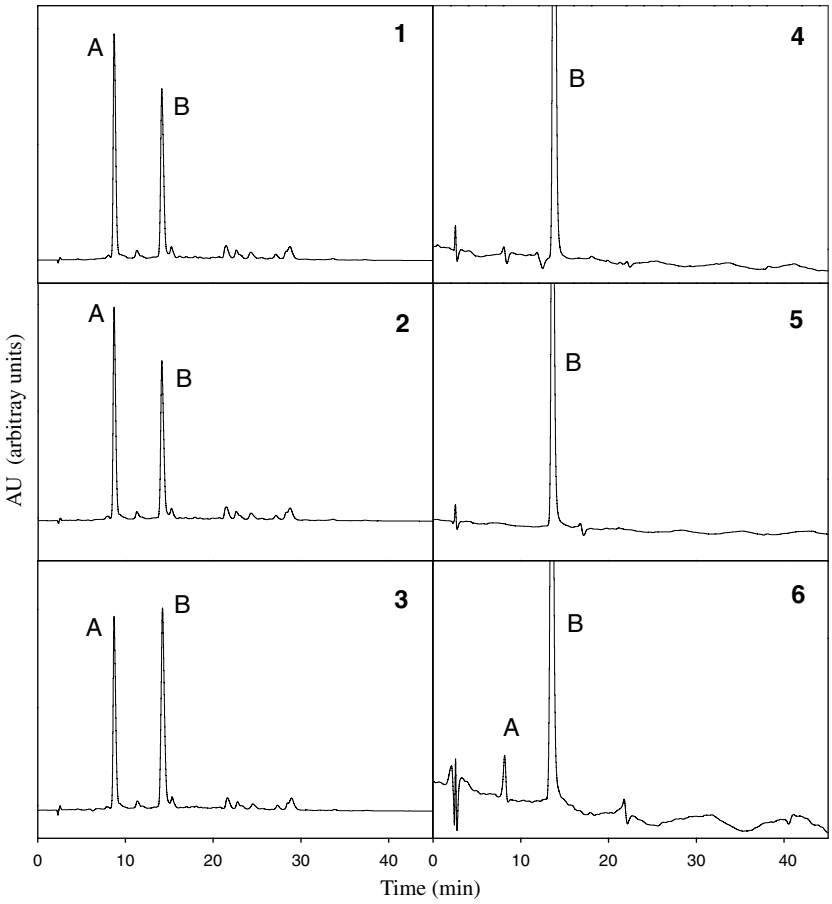

Fig. 1. HPLC chromatograms of caffeic acid derivatives, neochlorogenic acid (peak A) and chlorogenic acid (peak B) detected at 320 nm from: (1) original chokeberry juice, (2) chokeberry juice subjected to 2 h of simulated gastric digestion with HCl-pepsin at pH 2.0, (3) chokeberry juice after further 2 h of digestion with pancreatin-bile salts at pH 7.5, (4) standard solution of commercial chlorogenic acid, (5) chlorogenic acid after stomach simulation and (6) chlorogenic acid after further incubation with the pancreatin–bile salts mix.

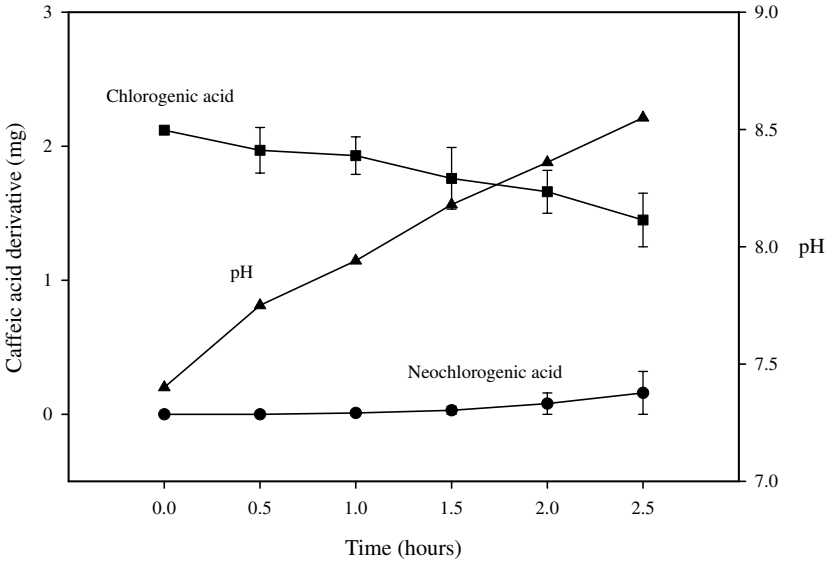

Fig. 2. Time course for the stability of chlorogenic acid (■–■) and the formation of neochlorogenic acid (●–●) during simulated pancreatic digestion (pancreatin-bile salts at pH 7.5). Data are expressed as mean quantities (total mg in the incubation mixture) (±SD). Variation in the pH of the incubation mixture during digestion is also shown (▲–▲).

genic acid, indicating the lack of esterase activity in the commercial pancreatin used in this study.

Importantly, control digestions of chokeberry juice or of pure compounds in the absence of enzymes (pepsin or pancreatin + bile salts) showed similar results for all the examined phenolic compounds (data not shown) as for those obtained in the presence of enzymes indicating that the observed effects were mostly due to the chemical conditions of the assay.

4. Discussion

In this study, we performed a simulated stomach and duodenum digestion of a chokeberry juice rich in polyphenols to determine the stability of these compounds under the incubation conditions. As stated in the Materials and methods section, the *in vitro* digestion was carried out by following a previously published method (Gil-Izquierdo et al., 2001) but with some modifications. In the reported protocol, a dialysis membrane was used during the pancreatic digestion of orange juice and the dialyzation rates for several flavonoids were determined as an estimate of the availability for absorption of these compounds (Gil-Izquierdo et al., 2001). In a more recent report, the same method was used to assess bioavailability of raspberry anthocyanins, where the dialyzed compounds were considered representative of material that enter the serum and, the solution outside the membrane as the materials remaining in the lumen (McDougall et al., 2005). During the set up of the present in vitro digestion conditions, we observed substantial losses of some of the phenolic compounds when using a dialysis membrane. These losses can largely be attributed to the difficulty of washing the membrane and recovering the compounds. In addition to these methodological problems, the use of a dialysis membrane as a means to estimate availability for absorption has, in our opinion, some limitations. Dialysis is a complex process affected by factors such as, volume and composition of the buffer used, sugar concentrations in the sample or ability of certain molecules to bind with the membrane (McPhie, 1971). All of these parameters can affect the dialyzation of a specific compound which cannot be truly associated with absorption of the compound *in vivo* (Gil-Izquierdo et al., 2001) or to levels of this compound in serum (McDougall et al., 2005). It has been recognized that separation by centrifugation or filtration, followed by analysis of soluble compounds recovered after digestion of a food sample, is a good estimate of those compounds that have been released from the food matrix into the digestive juice and that become bioaccessible (Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005). In the present study, we eliminated the use of a dialysis membrane during the pancreatic digestion and simply determined total soluble phenolics recovered in the filtered digested samples as an estimate of compounds that may be available for uptake.

We also performed the *in vitro* digestion in the absence of light and flushing of the incubation mixture with N_2 for a few minutes to reduce the levels of dissolved O_2. When food is swallowed, it would be initially equilibrated with the O_2 tension of room air (160 Torr) (He et al., 1999). The levels of dissolved O_2 may even increase with mastication (Kanner & Lapidot, 2001). However, on passage to the stomach and later to the small intestine, the levels of O_2 would fall as O_2 diffuses across the mucosal membrane and a gradual process of equilibration with the capillary levels of O_2 (5–10 Torr) would occur, reaching values in the range of 50–60 Torr in the stomach and even lower in the mid-duodenum (He et al., 1999). When examining the effects of *in vitro* digestion on dietary polyphenols, light and O_2 are two important factors to consider as they can alter the structure and properties of phenolic compounds, due to oxidative degradation (Jorgensen, Marin, & Kennedy, 2004) and polymerization reactions (Talcott & Howard, 1999). These effects, however, might be diminished inside the gastrointestinal tract as compared to those *in vitro* models where digestion has been performed while exposed to atmospheric O_2 and light (open flasks) (Gil-Izquierdo et al., 2001; Perez-Vicente et al., 2002; Vallejo et al., 2004; Zhu et al., 2002; McDougall et al., 2005; Boyer et al., 2005; Walsh et al., 2003; Yokomizo & Moriwaki, 2005). A recent report showed that constant flushing with N_2 had no effect on the recoveries of quercetin and quercetin-3-glucoside during *in vitro* digestion of onions and apples (Boyer et al., 2005). However, we found a better recovery of anthocyanins in the absence of light and after flushing the samples with N_2 for a few minutes. *In vitro* systems with reduced levels of O_2 may be more representative of the physiological conditions in the stomach and small intestine than is digestion performed in open-air flasks.

Overall, our results on the stability of the major polyphenols in chokeberry juice during simulated gastric and pancreatic digestion are in line with previous reported findings which are summarized in Table 3. Despite some differences in the incubation conditions, most dietary polyphenols appear to be quite stable during gastric digestion as no significant changes have been reported in the flavonoids and phenolic acid contents of various foods incubated under conditions mimicking those that must occur in the stomach. Our results on the stability of anthocyanins under acidic conditions are in agreement with those reported for raspberry (McDougall et al., 2005) or pomegranate (Perez-Vicente et al., 2002) anthocyanins. We and others (Perez-Vicente et al., 2002) reported a small increase in anthocyanins after *in vitro* stomach digestion. This fact has been attributed to the lower pH of the sample after the gastric incubation (pH 2.0) than the pH of the fresh initial sample (pH ~3.4 for the initial chokeberry juice) which renders an increase of the flavylium cation in the solution (Perez-Vicente et al., 2002). The high stability under the stomach conditions of flavonols or flavan-3-ols is also comparable to previous reported *in vitro* (Boyer et al., 2005; Zhu et al., 2002) and *in vivo* (Rios et al., 2002) stabil-

Table 3
In vitro stability of some dietary polyphenols after gastric and intestinal simulated digestion

Food	Phenolic compounds	*In vitro*[a] gastric conditions	Stability results	*In vitro*[a] intestinal conditions	Stability results	References
Orange juice	Flavanones	Pepsin–HCl, pH 2.0, 2 h	No changes detected	Pancreatin–Bile, $NaHCO_3$, pH 7.5, 2 h	50–60% conversion into chalcones.	Gil-Izquierdo et al., 2001
Pomegranate juice	Anthocyanins	Pepsin–HCl, pH 2.0, 2 h	10% increase	Pancreatin–Bile, $NaHCO_3$, pH 7.5, 2 h	About 80% decrease	Perez-Vicente et al., 2002
Cocoa	(−)-Epicatechin, (+)-Catechin, Dimers B_2, B_5	NaCl–HCl, pH 1.8, 1 h	Monomers stable. Isomerization and degradation of dimers	$NaHCO_3$, pH 8.5, Several hours	Almost complete degradation between 2 and 8 h	Zhu et al., 2002
Broccoli	Flavonoids, hydroxycinnamoyl derivatives	Pepsin–HCl, pH 2.0, 2 h	Flavonoids stable. 6 to 25% losses of cinnamics	Pancreatin–Bile, $NaHCO_3$, pH 7.5, 2 h	About 80–85% losses	Vallejo et al., 2004
Soy bread	Isoflavonoids	Pepsin–HCl, pH 2.0, 1 h, N_2	No changes detected	Pancreatin–Bile, $NaHCO_3$, pH 6.9, N_2, 2 h	Isoflavonoids mostly stable. Some conversion to aglycones	Walsh et al., 2003
Raspberry	Anthocyanins	Pepsin–HCl, pH 2.0, 2 h	No changes detected	Pancreatin–Bile, $NaHCO_3$, pH 7.5, 2 h	30% losses	McDougall et al., 2005
–	Myricitrin	NaCl–HCl, pH 1.8, 1 h	No changes detected	$NaHCO_3$, pH 8.5, 1 h	Almost complete degradation	Yokomizo and Moriwaki, 2005
Onions, apples	Quercetin, Quercetin-3-glucoside	Pepsin–HCl, pH 2.0, 30 min	No changes detected	Pancreatin–Bile, $NaHCO_3$, pH 6.5, 1 h	50–75% loss of quercetin, 10% loss of quercetin-3-glucoside	Boyer et al., 2005

[a] All *in vitro* assays were performed at 37 °C.

ity. Stabilities of polyphenols under gastric conditions is in accordance with early absorption from the stomach of intact anthocyanin glycosides (Talavera et al., 2003) or chlorogenic acid (Lafay et al., 2006).

A good proportion of most of the dietary polyphenols examined is, however, lost during incubation with pancreatin-bile salts at neutral or slightly basic pH. Anthocyanins are largely affected by the alkaline conditions of the pancreatic digestion with reported *in vitro* recoveries between 70% and 20% (McDougall et al., 2005; Perez-Vicente et al., 2002). In a recent *in vivo* study, the recovery of anthocyanins from the gastrointestinal tract of pigs fed a raspberry meal was reported to vary, approximately from 40% to 80 %, depending on the sugar moiety (Wu et al., 2006). Among the anthocyanins examined, cyanidin-3-glucoside exhibited the lowest recovery (only about 2%) and this was not associated with a parallel increase in detection of the compound or its metabolites in urine, indicating a possible degradation of the compound in the pig gut (Wu et al., 2006). We recovered ~57% of cyanidin-3-glucoside after *in vitro* pancreatic digestion of the chokeberry juice. The differences may be attributed to factors such as food matrix (chokeberry juice *vs.* raspberry freeze-dried powder mixed with water) but other factors may be affecting the compounds *in vivo*. Flavonols in chokeberry juice are also partially degraded under *in vitro* intestinal conditions, with recoveries of individual compounds ranging between 70% and 85%. We detected some differences between the stability of pure quercetin-3-rutinoside and the stability of this compound in the chokeberry during digestion. The pure compound was more stable when incubated alone than was the same compound in the chokeberry juice (3% losses of the pure compound *vs.* 30% losses of quercetin-3-rutinoside in the chokeberry juice). Similar differences have been reported for pure quercetin and quercetin in onion (Boyer et al., 2005) and, although these results cannot easily be explained, it has been suggested that interactions with other compounds in the food matrix can influence and alter stability during digestion.

Our results on the caffeoylquinic derivatives are in disagreement with those described previously for these two compounds in digested broccoli where high losses for these compounds were reported and attributed to the chemical conditions of the assay (Vallejo et al., 2004). We have shown a greater stability for these two compounds under the present *in vitro* conditions which differ from the reported ones in which no dialysis membrane was used and the incubations were performed in the absence of O_2 and light, factors that may have affected the results in the broccoli digestion. Moreover, the effect of the food matrix cannot be disregarded. We also observed a decrease in neochlorogenic acid, accompanied by an increase in chlorogenic acid, in digested chokeberry. We did not have pure commercial neocholorogenic acid but, instead, we incubated pure chlorogenic acid under the current *in vitro* conditions and found some conversion to neochlorogenic. These results, and previous reports on isomerization between these two compounds under certain conditions (Farah, De Paulis, Trugo, & Martin, 2005), suggest that, during the pancreatic incubation, some isomerization between neochlorogenic acid and chlorogenic acid may occur. However, we can not disregard other causes for the observed changes, such as some degradation of neochlorogenic acid due to the presence of some residual polyphenol oxidase activity (Gonzalez, de Ancos, & Cano, 2000), which may act prevalently on neochlorogenic acid (Del Caro, Piga, Pinna, Fenu, & Agabbio, 2004).

Our results, and previous reports, confirm that dietary polyphenols are highly sensitive to the mild alkaline conditions in the small intestine and suggest that, during digestion in the duodenum, a proportion of these compounds may be transformed into different structural forms with different chemical properties. After thorough examination of the HPLC–DAD and HPLC-MS–MS chromatograms from original and digested chokeberry samples, we were not able to detect any new peak(s) that might have suggested the formation of any derivatives after pancreatic digestion. Some of these putative derivatives might not have been detected under our current analytical conditions. The nature of some chemical derivatives formed from some flavonoids has been known for some time. For example, oxidation of quercetin yields *ortho*-quinone and transient quinone methide adducts which are likely to play an important role in quercetin bioavailability and bioactivity (Rietjens et al., 2005). It has also been well described that, at pH > 4.5, the stability of anthocyanins decreases rapidly as the red flavylium cation is transformed into other less stable forms, such as the colourless pseudobase (pH 4–5; $M^+ = 304$), the purple quinoidal base (pH 6–7; $M^+ = 286$) and the yellow chalcone (pH 7–8; $M^+ = 304$), which eventually lead to degradation of the anthocyanins (Nielsen, Haren, Magnussen, Dragsted, & Rasmussen, 2003; Fleschhut et al., 2005). Under our analytical conditions, HPLC-MS-MS analysis of a cyanidin-3-glucoside standard solution, both at pH 2.0 and pH 8.0, clearly showed the decrease of the cyanidin-3-glucoside peak under alkaline conditions and the formation of two other peaks with masses $M^+ = 487$, 303, 257 and $M^+ = 489$, respectively. We were not able to detect any of these peaks in the chromatograms from the pancreatic digestion of chokeberry. Some other polyphenol derivatives have been recently described. Dehydrodimers of flavan-3-ols can be formed from the oxidation of (+)-catechin and (−)-epicatechin (Sun & Miller, 2003). A dimeric quercetin oxidation product (Cherviakovsky et al., 2006), anthocyanin-pyruvic acid adducts (Faria et al., 2005) and some putative dimers and phenolic compounds derived from anthocyanidins (Aura et al., 2005; Fleschhut et al., 2005) have been reported. Among the phenolic compounds, protocatechuic acid can be formed by spontaneous degradation from cyanidin (Fleschhut et al., 2005). We detected trace quantities of protocatechuic acid (λ_{max} (nm): 259.9/294.2; m/z^- 153.0) in the original chokeberry juice which increased slightly during the pancreatic digestion (data not shown).

It is tempting to speculate that, after gastric and pancreatic digestion of the chokeberry juice, as well as of any polyphenol-containing foods, some of these oxidation and degradation products may be formed *in vitro* and *in vivo*. This is an important issue that needs further and thorough investigation as these derivatives may have a major impact on the bioaccessibility, bioavailability and biological activity of polyphenols.

With all the limitations of *in vitro* models, and all caution when interpreting the results derived from them, methods mimicking *in vivo* human conditions, such as those simulating the gastrointestinal digestion remain a useful tool for investigating the effects that this essential physiological process may have in the fate of specific food components such as polyphenolic compounds. However, there are important discrepancies between *in vitro* and *in vivo* results, such as those reported for the dimeric flavan-3-ols, B2 and B5, which are degraded to a great extent after 1 h incubation in simulated gastric juice at pH 1.8 (Zhu et al., 2002) but remain stable in the human stomach (Rios et al., 2002). These differences indicate that *in vitro* methods need to be further improved and validated with more *in vivo* animal and human studies.

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